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Sec3p Is a Spatial Landmark for Polarized Secretion in Budding Yeast

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Summary

Exocytosis in yeast occurs at plasma membrane subdomains whose locations vary with the cell cycle, but the primary protein determinants of these sites are unknown. A functional fusion of Sec3 protein with green fluorescent protein (Sec3-GFP) localizes to the site of polarized exocytosis for each cell-cycle stage, where it colocalizes with Sec4p and Sec8p. Sec3-GFP localization is independent of secretory pathway function, of the actin and septin cytoskeletons, and of the polarity establishment proteins. We propose that Sec3p is a spatial landmark defining sites of exocytosis. Polarized secretion would result from the coupling of actin-dependent vesicle targeting with Sec3p-dependent establishment of the vesicle fusion site.

Introduction

The establishment of polarized secretory pathways is crucial to the development of many different cell types (reviewed in Drubin and Nelson, 1996). Polarized epithelia, for example, require distinct apical and basolateral plasma membrane domains for their functions in transport of ions and other solutes (reviewed in Simons, 1995). The budding yeast *Saccharomyces cerevisiae* uses polarized secretion for bud growth, cytokinesis, and response to mating pheromone (reviewed in Govindan and Novick, 1995).

Exocytosis in yeast occurs at distinct plasma membrane subdomains, which vary with the cell cycle. Unbudded cells polarize secretion to the new bud site prior to bud emergence and continue to direct secretion to the tip of the emerging bud (Tkacz and Lampen, 1972, 1973; Farkas et al., 1974; Field and Schekman, 1980). When the daughter cell is about two-thirds the size of the mother, secretion becomes isotropic over the surface of the daughter cell, whereas very little growth or secretion occurs in the mother cell. Following nuclear division, the secretory pathway orients to the mother-bud neck, resulting in cytokinesis and septation (Byers, 1981). Yeast responding to mating pheromone direct secretion to the tips of mating projections (Tkacz and Lampen, 1973; Field and Schekman, 1980). A polarized secretory pathway necessitates that appropriate sites for vesicle docking and fusion be defined by components of the different target membranes, but the identity of these components is unclear.

In yeast, a polarized secretory pathway is thought to be generated by a hierarchical cascade of events (reviewed in Drubin and Nelson, 1996). First, a bud site is chosen through the action of the *BUD* genes according to haploid- or diploid-specific programs (Chant and Pringle, 1995). This spatial signal is then interpreted by the polarity establishment proteins, such as the rho family GTPase Cdc42p (Johnson and Pringle, 1990). The polarity establishment proteins regulate polarization of the actin cytoskeleton (Li et al., 1995), which in turn is thought to establish a polarized secretory pathway. Among the exceptions to this simple hierarchical scheme are three yeast proteins required for exocytosis, Sec3p, Sec4p, and Sec9p, which also have roles in development of cell polarity. Diploids that have homozygous mutations in the genes encoding any of these proteins establish new bud sites in random positions, rather than in the normal bipolar pattern (Haarer et al., 1996; Finger and Novick, 1997). Some of these mutants, especially the more severe alleles of *sec3*, also have wide necks and somewhat depolarized chitin deposition, indicating that they may be defective in polarizing secretion (Finger and Novick, 1997). The requirements for these proteins in both polarity establishment and in exocytosis suggests that one or more of them could act as the primary protein determinant of the site for polarized exocytosis. A protein that functions as a spatial landmark for the site of exocytosis should be found on the target membrane specifically at sites of vesicle fusion, and its localization should be independent of ongoing membrane traffic. The localization of Sec4p and Sec9p eliminates these proteins as exocytosis site determinants. Sec4p, a rab family GTPase (Salminen and Novick, 1987), localizes to post-Golgi secretory vesicles (Goud et al., 1988; Walch-Solimena et al., 1997) and Sec9p, a t-SNARE, is found on the plasma membrane in both mother and daughter cells (Brennwald et al., 1994).

Sec3p, a 155 kD protein with a 145 amino-acid coiled coil (Haarer et al., 1996; Finger and Novick, 1997), is the remaining candidate for an exocytosis site determinant. It has been found as a component of a large protein complex, which also includes Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p (TerBush et al., 1996). A homologous complex has been isolated from mammalian brain, although all of the components are not yet defined (Ting et al., 1995; Hsu et al., 1996; Guo et al., 1997; Hazuka et al., 1997; Kee et al., 1997). Sec8p, a component of the yeast complex, has been localized to tips of small-budded cells, a site of polarized exocytosis (TerBush and Novick, 1995). To ascertain whether Sec3p could be one of the primary determinants of the site of polarized exocytosis, we created a functional fusion of green fluorescent protein (GFP) to the carboxyl terminus of Sec3p, and we used Sec3-GFP to explore changes in the localization of this protein over time and to test the dependence of this localization on secretory, cytoskeletal, and polarity establishment proteins. Sec3-GFP localizes to the sites of exocytosis at each stage of the cell cycle. The localization is independent of secretory pathway function (including other complex

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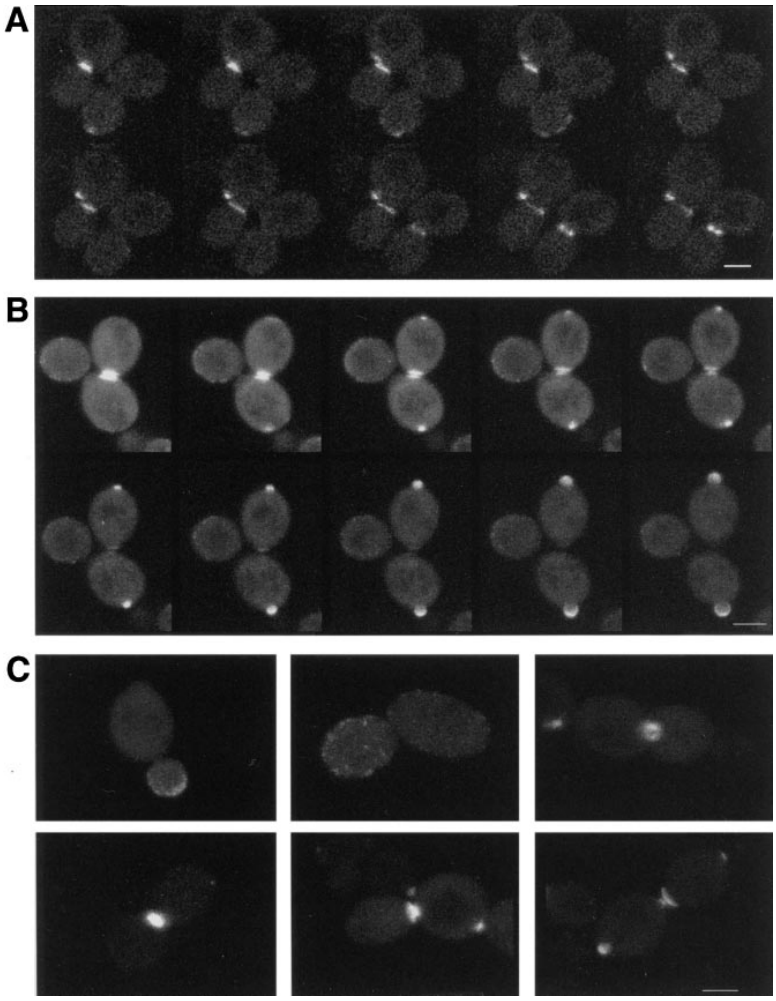

Figure 1. Sec3-GFP Localization in Living Cells

(A) Time lapse confocal images of Sec3-GFP in haploid (FY110) cells. Panels (left to right), 2 min apart.

(B) Time lapse confocal images of Sec3-GFP in diploid (NY648) cells, showing establishment of new bud sites in mother and daughter cells following cytokinesis. Panels (left to right), 3 min apart.

(C) Images from 3-D projections of confocal Z-series, showing different points in the cell cycle of diploid (NY1262) cells. Top row, left to right: small-budded cell with Sec3-GFP at the bud tip; large-budded cell with patches of Sec3-GFP in the bud; cell at cytokinesis with a ring of Sec3-GFP at the mother-bud neck. Bottom row, left to right: filled-in ring of Sec3-GFP at mother-bud neck; new bud site being established and small satellite spot of Sec3-GFP adjacent to mother-bud neck; cells with Sec3-GFP at both poles. Scale bars, 3 μM.

components), independent of the actin and septin cytoskeletons, and possibly independent of polarity establishment proteins such as Cdc24p and Cdc42p. From these results, we propose that Sec3p acts as a spatial landmark to help establish the site of polarized exocytosis in yeast. Efficient polarized secretion would therefore result from the coupling of an actin-dependent pathway for targeted delivery of vesicles with the actin-independent localization of Sec3p to the docking/fusion site.

Results

Localization of Sec3-GFP in Living Cells

To investigate the localization of Sec3p in living cells, a low-copy number plasmid, pSEC3-GFP3-1 that inserted the *GFP* coding sequence in frame with the 3′ end of the *SEC3* coding sequence was constructed. This construct expressed a fusion protein of Sec3p with GFP fused to its carboxyl terminus under the control of the endogenous *SEC3* promoter, so that the Sec3-GFP would be present in physiological quantities. This plasmid complemented all three temperature-sensitive (ts) mutant alleles of *sec3* (*sec3-2*, *sec3-4*, and *sec3-5*) that were tested for growth at the restrictive temperature of 37°C. Another low-copy number plasmid, pGFP3-1, was constructed, which replaced the *SEC3* coding sequence with that of *GFP*. Both plasmids were transformed into wild-type and *sec3* mutant haploids and diploids, and the localization of the GFP was monitored by confocal microscopy of living cells. The cells expressing GFP alone exhibited fluorescence throughout the cytoplasm except for the vacuole (data not shown). Cells expressing Sec3-GFP displayed a different pattern of fluorescence, which changed dynamically over the course of the cell cycle (Figure 1). In haploid cells following cytokinesis, Sec3-GFP was localized to both sides of the mother-bud neck. Prior to bud emergence, the Sec3-GFP rapidly (3–5 min) relocalized to the new adjacent bud site as the old concentration disappeared and remained at the bud tip as the bud emerged in small-budded cells. As the bud enlarged, the Sec3-GFP localization became patchier, although still at the bud cortex, and then at cytokinesis, the Sec3-GFP rapidly (3–5 min) concentrated at both sides of the mother-bud neck, appearing initially to be a ring that subsequently filled in (Figure 1A). The localization in diploids was similar in most respects, except that the bud sites chosen were usually distal, and remnants of the old localization remained for a considerable time after establishment of

the new bud site (Figure 1B). Unbudded cells with concentrations of Sec3-GFP at both poles were frequently seen (Figures 1B and 1C), implying that the cell may be secreting from both ends simultaneously, which could perhaps give rise to the characteristic lemon shape of diploid cells. A spot of Sec3-GFP adjacent to the former neck concentration was sometimes seen in small-budded cells where the bud was at the opposite pole (Figure 1C). Sec3-GFP also localized to the mating projections of cells responding to mating pheromone (Figure 3C). Sec3-GFP therefore localized to the site of polarized secretion appropriate to each stage of the cell cycle.

Because the fluorescence intensity of GFP should directly correlate with the concentration of Sec3-GFP, we used the fluorescence of Sec3-GFP in cells where the endogenous *SEC3* gene(s) were tagged with GFP to compare the amount of fluorescence localized to that in the general cytoplasm. We restricted our analysis to those cells undergoing cytokinesis, in which Sec3-GFP is found localized only at the well-defined mother-bud neck. By these estimates, from 17%–30% of the Sec3-GFP in haploid cells is localized to the neck, with the remainder cytoplasmic. These figures are in good agreement with the estimates of the amounts of the Sec3/5/6/8/10/15/Exo70p complex associated with the plasma membrane from fractionation studies using haploid cells (Bowser and Novick, 1991; Bowser et al., 1992). In diploid yeast, from 10%–25% of Sec3-GFP is concentrated at this site. We have previously shown that overexpression of *SEC3* in a wild-type background does not affect cell growth or morphology (Finger and Novick, 1997). The localization of Sec3-GFP also does not dramatically change upon overexpression, although a modest increase in the proportion that is cytoplasmic is seen. When Sec3-GFP is expressed from a low-copy number plasmid, approximately 20% of the total is found at the mother-bud neck at cytokinesis, whereas only 10% of the total Sec3-GFP expressed from a high-copy number plasmid is found at mother-bud necks in cytokinesing cells.

Photobleaching experiments suggest that Sec3-GFP stably associates with the plasma membrane. When the cytoplasm was bleached, there was no detectable exchange between the unbleached pool at the bud tip and the cytoplasmic pool over the 10 min course of the experiment, indicating that Sec3-GFP remains plasma membrane associated for at least 10 min (data not shown).

Sec3-GFP Colocalizes with Sec4p and with Sec8-3Xmyc

To determine whether Sec3-GFP colocalized with other components of the secretory pathway that have a polarized localization, we performed indirect immunofluorescence localization of Sec4p in conjunction with the direct visualization of Sec3-GFP. Sec4p has been previously shown to localize to the tips of small-budded cells (Novick and Brennwald, 1993), and by immunoelectron microscopy, Sec4p is concentrated on post-Golgi secretory vesicles (Walch-Solimena et al., 1997). Sec3-GFP and Sec4p showed considerable overlap in their localization (Figure 2A). Sec4p appeared to extend further back toward the mother cell in small-budded cells, but when Sec3-GFP and Sec4p showed punctate localization in large-budded cells, the patches of Sec3-GFP and of Sec4p immunoreactivity appeared to exactly coincide. In unbudded and small-budded diploid cells, Sec3-GFP and Sec4p colocalized to both poles of the cell, substantiating the idea that secretion in diploids can occur simultaneously from both poles.

Sec8p, another component of the Sec3/5/6/8/10/15/Exo70p complex, was previously shown to localize to bud tips and prebud sites via indirect immunofluorescence of c-myc-tagged Sec8p, but it had not been detected at later cell-cycle stages (TerBush and Novick, 1995). We used the triple c-myc tagged Sec8p (TerBush et al., 1996), reasoning that its better immunoprecipitation properties might give improved indirect immunofluorescence. In addition to the localization described previously at prebud sites and at the tips of small buds (TerBush and Novick, 1995), we were also able to detect Sec8-3Xmyc at the mother-bud neck in cytokinesing cells and to demonstrate that Sec8-3Xmyc and Sec3-GFP colocalize (Figure 2B).

Sec3-GFP Localization Is Independent of a Functional Secretory Pathway

Post-Golgi vesicle formation can be abolished by blocking the secretory pathway at ER to Golgi or intra-Golgi stages of transport using ts secretory mutants that affect these stages. Polarized localization of Sec4p is lost in these conditions within 10 min of temperature shift (Walch-Solimena et al., 1997). We transformed pSEC3-GFP3-1 into secretory mutants affecting ER to Golgi and intra-Golgi transport. If Sec3-GFP localized to the bud tip either via localization on secretory vesicles or by recruitment to the target membrane in response to vesicle docking, the abolishment of vesicle formation would result in the loss of Sec3-GFP concentrations from sites of polarized exocytosis. In contrast, in all of the ER to Golgi and intra-Golgi *sec* mutants examined, Sec3-GFP remained localized to the sites of polarized secretion even after a 1 hr shift to the restrictive temperature (Figure 2C, Table 1). This suggests that Sec3-GFP stably associates with the target membrane at the site of exocytosis, in agreement with the photobleaching experiments.

We next examined the localization of Sec3-GFP in all of the other post-Golgi *sec* mutants. Again, we found that even after a 1 hr shift to the restrictive temperature of 37°C, Sec3-GFP remained localized to sites of polarized exocytosis (Figure 2C, Table 1). Among these mutants were *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, and *sec15-1*, which encode mutant forms of components of the complex with which Sec3p associates. Thus, Sec3-GFP was able to localize to the sites of polarized exocytosis in the absence of secretory pathway function at any stage of the secretory pathway.

Sec8-3Xmyc Localization Depends on a Functional Secretory Pathway

Because Sec3p localization is independent of the other components of the complex, we wanted to see if the

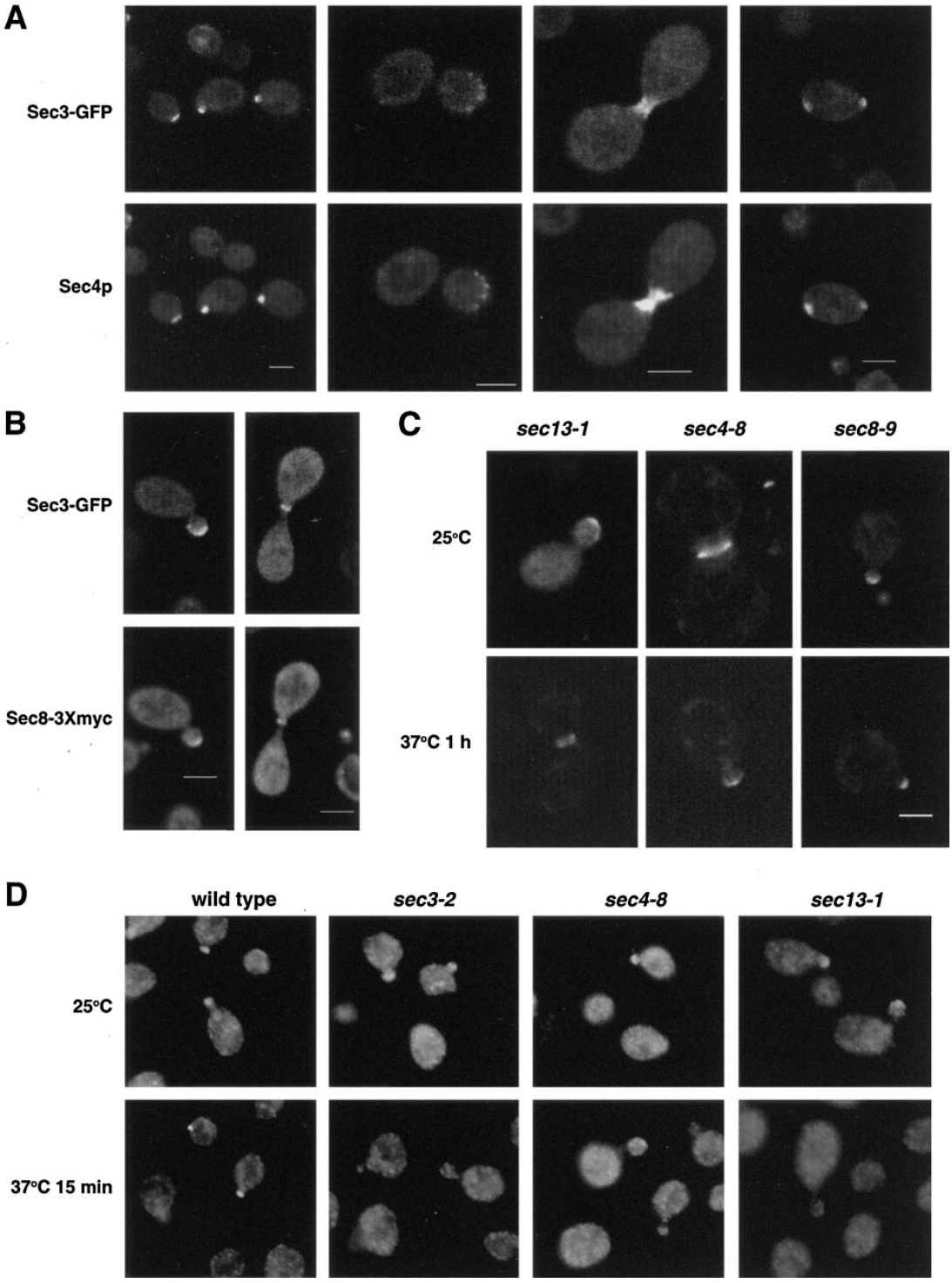

Figure 2. Sec8-3Xmyc, but Not Sec3-GFP, Requires a Functional Secretory Pathway for Localization

Confocal images of Sec3-GFP fluorescence with either indirect immunofluorescence of Sec4p ([A]; NY13, first three panels left to right; NY648, fourth panel) or Sec8-3Xmyc ([B]; NY1377) demonstrate colocalization of Sec3-GFP with both proteins. (C) Sec3-GFP maintains localization after 1 hr at 37°C in ts *sec* mutants including *sec4-8* (NY405), *sec8-9* (NY410), and *sec13-1* (NY414). (D) Sec8-3Xmyc localization is lost after 15 min at 37°C in ts sec mutants including *sec3-2* (NY1372), *sec4-8* (NY1373), and *sec13-1* (NY1438). Wild-type cells (NY1583) maintain Sec8-3Xmyc localization. Scale bars, 3 μM.

Table 1. Summary of Sec3-GFP and Sec8-3Xmyc Localization in Mutants

	Sec3-GFP			Sec8-3Xmyc		
Mutant	25°C	37°C 15 min	37°C 1 hr	25°C	37°C 15 min	37°C 1 hr
Wild-type	P	P	P (97%)	P (86%)	P (70%)	P (64%)
sec1-1	P	P	P (87%)	P (63%)	U/P (26%)	U (4%)
sec2-41	P	P	P (93%)	U/P (46%)	U (3%)	U (6%)
sec3-2	P	P	P	P (83%)	U/P (46%)	U (16%)
sec4-8	P	P	P (84%)	P	U	U
sec5-24	P	P	P (82%)	P (51%)	U (13%)	U (6%)
sec6-4	P	P	P (90%)	P	U/P	U
sec7-1	P	P	P	U/P	U	U
sec8-9	P	P	P (92%)	P	U	U
sec9-4	P	P	P (67%)	P	U	U
sec9-7	P	P	P (83%)			
sec10-2	P	P	P (88%)	P	U	U
sec13-1	P	P	P (73%)	P	U/P	U
sec14-3	P	P	P	P (84%)	U (2%)	U (7%)
sec15-1	P	P	P (84%)	U/P	U/P	U
sec16-2	P	P	P (73%)			
sec17-1	P	P	P	P	U	U
sec18-1	P	P	P	P	U	U
sec19-1	P	P	P	P (76%)	U (7%)	U (10%)
sec20-1	P	P	P			
sec21-1	P	P	P			
sec22-3	P	P	P	P (81%)	U (4%)	U (5%)
sec23-1	P	P	P			
ypt1-3	P	P	P	P	U	U
act1-2	P	P	P			
act1-3	P	P	P (89%)	P	U	U
pfy1-111	P	P	P			
myo1Δ	P					
myo2-66	P	P	P	P (73%)	U/P (32%)	P (53%)
myo3Δ	P					
myo5Δ	P					
myo3Δmyo5Δ	P					
cdc3-1[a]	P	P	P			
cdc10-10[a]	P	P	P			
cdc11-1[a]	P	P	P			
cdc12-1[a]	P	P	P			
cdc24-4[b]	P	P	P			
cdc42-1[b]	P	P	P			
bem3-1[b]	P	P	P			
bud3Δ	P					
cdc28-1[c]	P	P	U			

P, localization of the protein was polarized; U, localization of the protein was unpolarized; U/P, localization of the protein is partially depolarized. The proportion of cells with polarized Sec3-GFP or Sec8-3Xmyc is shown in parentheses.

[a] Cells still had polarized Sec3-GFP at 2 hr of shift.

[b] Cells still had polarized Sec3-GFP at 4 hr of shift.

[c] Loss of Sec3-GFP localization was first observed at 30 min of shift.

converse was true; that is, do other complex components require functional Sec3p for their localization? This is an intriguing possibility, because the complex appears to be completely disrupted in a *sec3-2* mutant background (TerBush and Novick, 1995). In the ts *sec3-2* strain, Sec8-3Xmyc became mislocalized, with the amount of mislocalization increasing with increased time at 37°C (Figure 2D, Table 1). Rather than localizing to the tips of small-budded cells, the Sec8-3Xmyc localized in a punctate cytoplasmic distribution. This effect was not specific to *sec3* mutants, because we found that the polarized distribution of Sec8-3Xmyc was lost after temperature shift in every *sec* mutant examined from both early steps (*sec7-1*, *sec13-1*, *sec14-2*, *sec17-1*, *sec18-1*, *sec19-1*, *sec22-3*, and *ypt1-3*) and post-Golgi stages (*sec1-1*, *sec2-41*, *sec3-2*, *sec4-8*, *sec5-24*, *sec6-4*, *sec9-4*, *sec10-2*, *sec15-1*) of secretion (Figure 2D, Table 1). This indicates that Sec8-3Xmyc localization depends on continuous secretory pathway function at all stages, in contrast to Sec3-GFP localization, which is independent of secretory pathway function.

Sec3p Does Not Colocalize with Actin, but Their Changes in Localization Are Temporally Coordinated

A functional actin cytoskeleton has been shown to be necessary for efficient polarized secretion (Novick and Botstein, 1985), and actin is concentrated in regions of active cell growth (Adams and Pringle, 1984). Sec3p and the actin cytoskeleton were functionally connected by the identification of genetic interactions between *SEC3* and *PFY1* (Haarer et al., 1996; Finger and Novick, 1997),

the gene encoding profilin (Haarer et al., 1990), which regulates actin assembly. Sec3p does not directly regulate the actin cytoskeleton (Finger and Novick, 1997). To investigate the possible connection between Sec3p and actin, we colocalized Sec3-GFP with actin by staining cells with rhodamine-labeled phalloidin (Figure 3A) or by indirect immunofluorescence with anti-actin antibodies (data not shown). In small-budded cells, Sec3-GFP localized to the edge of the bud tip, whereas actin was concentrated in cortical patches at the bud tip as well as in filaments oriented from mother to daughter. When the Sec3-GFP became localized in cortical patches in the bud, actin did as well, although these patches did not coincide. At cytokinesis (following nuclear division), Sec3-GFP localized to the membrane connection between the separating cells, and the actin appeared to be to either side of this connection. Finally, when a new bud site was being established, the concentration of Sec3-GFP at the prebud site appeared to emerge from the center of the ring of actin. Both techniques establish that the changes in Sec3-GFP localization occurred at the same points in the cell cycle as the changes in actin localization, but the two proteins did not appear to colocalize.

Sec3-GFP Localization Is Independent of the Actin Cytoskeleton

Because of the established involvement of the polarized actin cytoskeleton in post-Golgi secretion in yeast (Novick and Botstein, 1985; Ayscough et al., 1997; Mulholland et al., 1997; Walch-Solimena et al., 1997), we next investigated whether the localization of Sec3-GFP was dependent on a functional actin cytoskeleton. Sec3-GFP was localized in the ts actin mutants *act1-2* and *act1-3*, as well as in the ts profilin mutant *pfy1-111*, with which all known *sec3* alleles are synthetically lethal (Haarer et al., 1996; Finger and Novick, 1997). Sec3-GFP localization was also examined in strains containing mutations in four of the five yeast myosins, *myo1*Δ, *myo2-66*, *myo3*Δ, *myo5*Δ, and in the *myo3*Δ*myo5*Δ double mutant. Myo1p has been implicated in cytokinesis and septation (Watts et al., 1985; Rodriguez and Paterson, 1990), and the other myosins tested accumulate vesicles (Johnston et al., 1991; Govindan et al., 1995; Goodson et al., 1996). In all cases, the Sec3-GFP localization was maintained, either constitutively in the deletion strains or after a 1 hr shift to the restrictive temperature of 37°C for the ts mutants (Table 1). In the case of the *act1-3* mutation, the cells were stained with rhodamine-phalloidin to demonstrate the mutant actin morphology of loss of actin cables and depolarized actin patches. The rhodamine-phalloidin staining also demonstrated that the maintenance of Sec3-GFP localization is not due to residual polarized actin structures (data not shown).

To determine whether Sec3-GFP was able to achieve a new localization in the absence of actin function, we treated haploid MAT**a** cells with the mating pheromone α-factor. Cells thus treated arrest in G_1 and polarize to direct growth to the tip of mating projections, which are easily identified by light microscopy. Sec3-GFP is strongly localized to the tips of the mating projections. Cells released from the mating pheromone–induced cell-cycle arrest, quickly establish new bud sites that can be distinguished from the mating projection. When the *act1-3* mutant was simultaneously released from the α-factor arrest and shifted to the restrictive temperature of 37°C, Sec3-GFP was able to achieve a localization distinct from the mating projection (Figure 3B). Although concentrations of actin were seen in these cells, they usually remained at the mating projection and did not usually colocalize with the Sec3-GFP. This indicates the ability of Sec3-GFP to localize to a new site is not actin-dependent.

As a more stringent test of the ability of Sec3-GFP to localize independently of the polarized actin cytoskeleton, we observed localization of Sec3-GFP in cells recovering from α-factor arrest in the presence of the actin depolymerizing drug latrunculin-A (LAT-A). Although phalloidin staining demonstrated a complete absence of polymerized actin structures in LAT-A treated yeast, Sec3-GFP was well-localized in the cells thus treated (data not shown). Thus, maintenance of Sec3-GFP localization does not require a polarized actin cytoskeleton.

The most stringent test of a protein's ability to localize in the absence of polymerized actin, is to see whether cells released from G_0 (which are unpolarized) and treated with LAT-A, can localize the protein in question to the prebud site (Ayscough et al., 1997). We were unable to detect Sec3-GFP in cells exiting from G_0, presumably due to slow formation of the chromophore (Heim et al., 1994). We therefore used a strain where all of the Sec3p was C-terminally tagged with four consecutive HA epitopes and localized the Sec3-4XHA by indirect immunofluorescence. After 2 hr of treatment with LAT-A, 51% of cells displayed Sec3-4XHA concentrated at one pole, similar to untreated cells in both timing and proportion of cells (57%) with polarized Sec3-4XHA (Figure 3C). Cells were also stained in parallel with anti-actin and anti-Sec4p antibodies (data not shown). The actin staining revealed no normal actin structures; however, actin bars were seen. Sec4p staining revealed puncta distributed throughout the cytoplasm. The actin and Sec4p phenotypes were as described previously for LAT-A treated cells (Ayscough et al., 1997). Thus, by all criteria examined, the polarized localization of Sec3p is actin-independent.

In contrast, Sec8-3Xmyc was unable to maintain localization in the *act1-3* mutant at the restrictive temperature (Table 1), consistent with the inability of Sec8-3Xmyc to localize in LAT-A treated cells (Ayscough et al., 1997). Sec8-3Xmyc was able to maintain localization in the *myo2-66* mutant (Table 1), which does not appear to block exocytosis (Govindan et al., 1995).

Sec3-GFP Does Not Colocalize with Septins or Require Septins to Maintain Localization to the Mother-Bud Neck

Because Sec3-GFP localizes to the mother-bud neck at cytokinesis, it is possible that this localization might be mediated through interaction with the septins, Cdc3p, Cdc10p, Cdc11p, and Cdc12p, which are the putative components of filaments at the mother-bud neck that are necessary for cytokinesis (Longtine et al., 1996). To address this question, indirect immunofluorescence

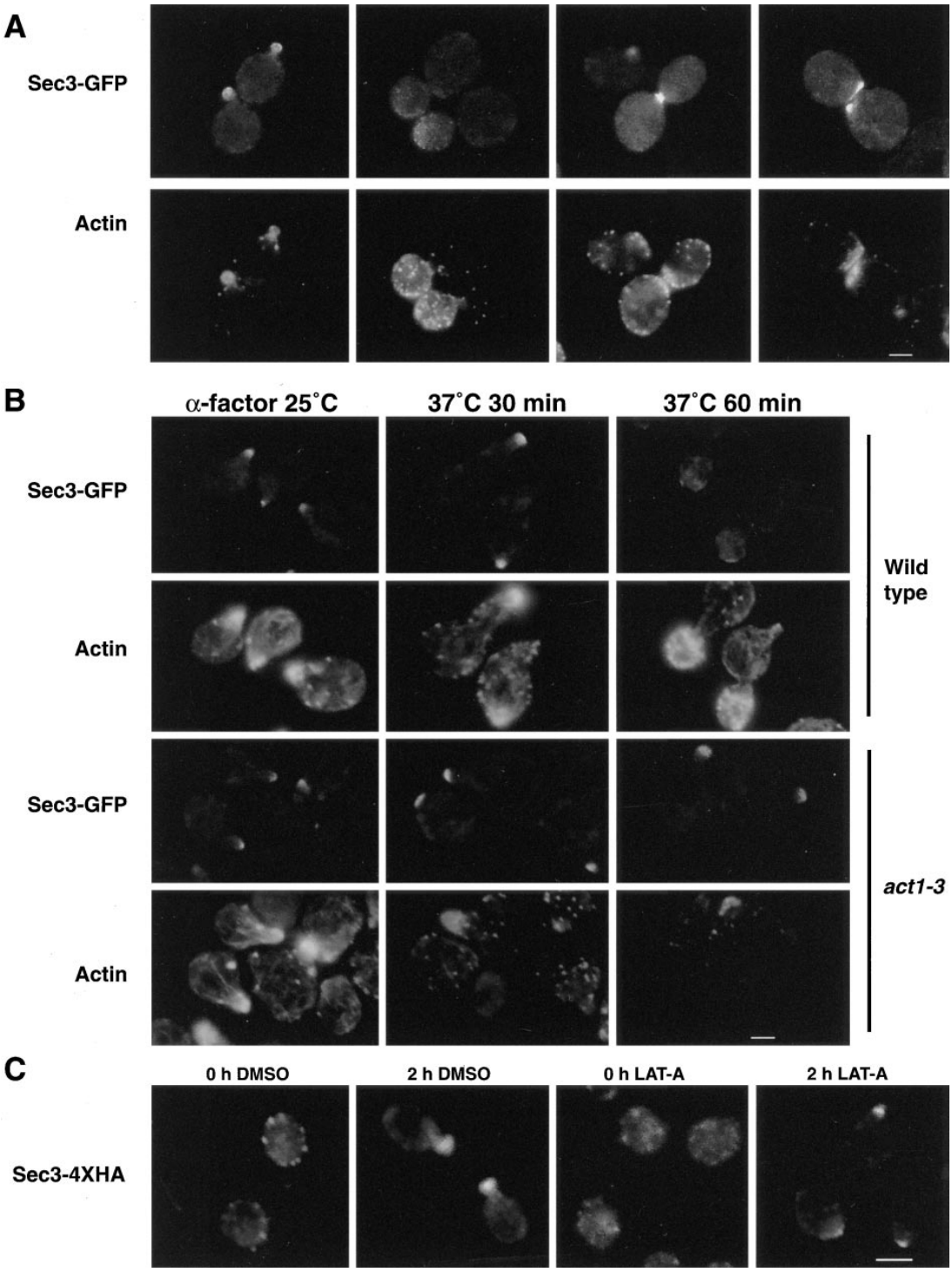

Figure 3. Sec3p Localization Is Independent from the Actin Cytoskeleton

(A) Sec3-GFP (top row) compared with actin (bottom row) localized by rhodamine-phalloidin staining (FY126) demonstrates lack of colocalization. Panels from left to right show small-budded cells, medium-budded cells, cells at cytokinesis, and postcytokinesis relocalization to new bud sites.

(B) Sec3-GFP localizes to new bud sites in wild-type (NY13) and *act1-3* (NY279) cells recovering for 1 hr at 37°C from a 2 hr α-factor arrest.

(C) Sec3-4XHA polarizes in FY140 cells growing out from G_0 in the presence of LAT-A for 2 hr at 25°C. Scale bars, 3 μM.

was used to localize Cdc3p tagged with the c-Myc epitope, in conjunction with Sec3-GFP visualization (Figure 4A). At cytokinesis the septin rings were to either side of the cytoplasmic bridge connecting the cells, where Sec3p localizes. In an unbudded cell, the Sec3-GFP was seen to form a patchy ring inside the ring of Cdc3p,

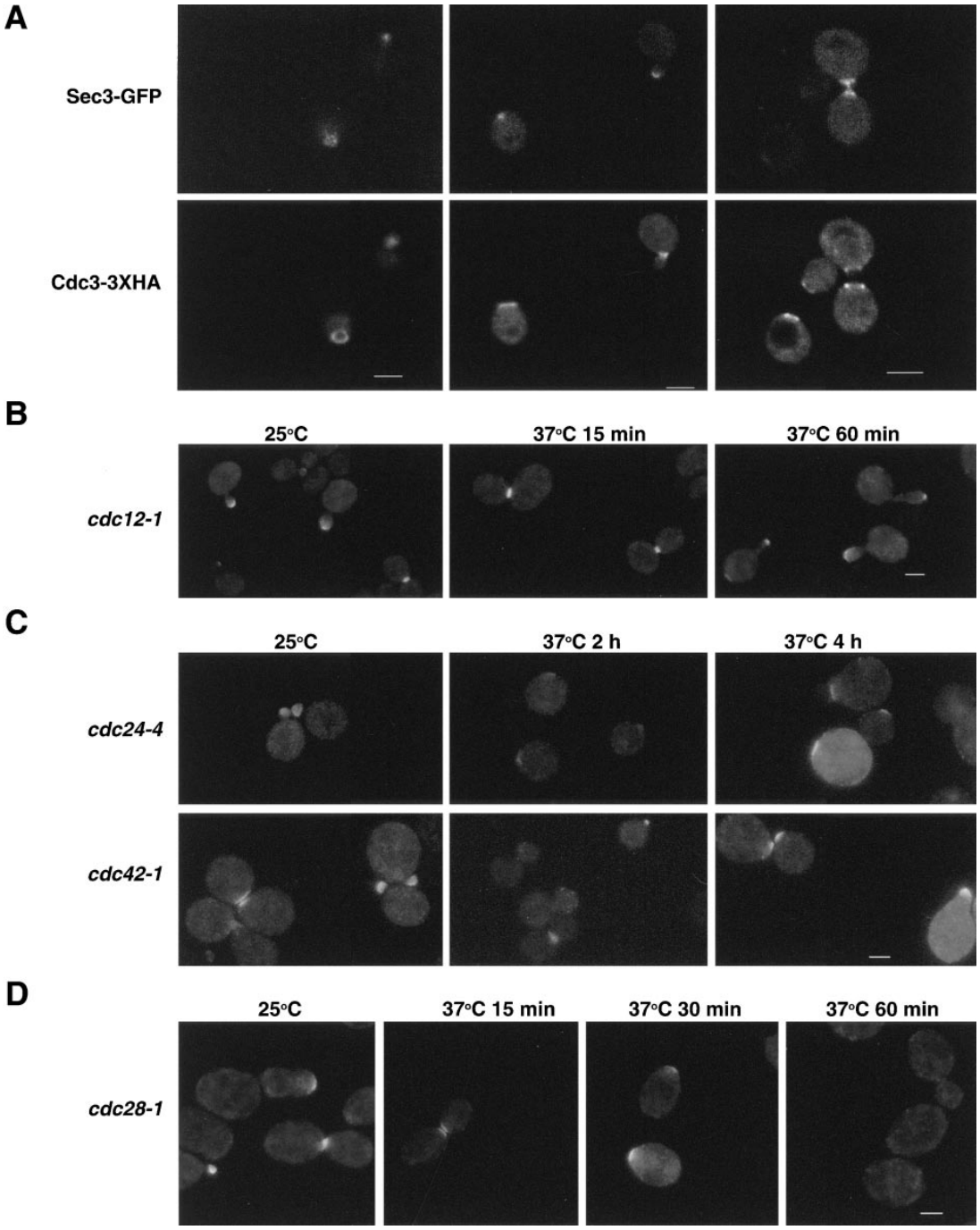

Figure 4. Sec3-GFP Localization in Morphogenetic and Cell-Cycle Mutants

(A) Confocal microscopy shows lack of colocalization of Sec3-GFP and Cdc3-3XHA in NY1358 in unbudded, small-budded, and cytokinesing cells.

(B) Sec3-GFP localization in the septin mutant *cdc12-1*. After 15 min at 37°C, Sec3-GFP can still be detected at the necks of large-budded cells. At later times (1 hr), Sec3-GFP is concentrated solely in bud tips.

(C) Sec3-GFP maintains polarized localization in the polarity establishment mutants *cdc24-4* (NY1442) and *cdc42-1* (NY1443) after prolonged shifts (2 and 4 hr) to 37°C.

(D) Polarized localization of Sec3-GFP is lost in the *cdc28-1* mutant within 1 hr at 37°C. Scale bars, 3 μM.

which was left over from cytokinesis. In cells at other stages of the cell cycle, again no colocalization was seen. At no stage of the cell cycle do Sec3-GFP and septins colocalize.

To ascertain whether the localization of Sec3-GFP at the mother-bud neck requires neck filament function, Sec3-GFP was expressed in the ts septin mutants *cdc3-1*, *cdc10-10*, *cdc11-1*, and *cdc12-1*. These strains

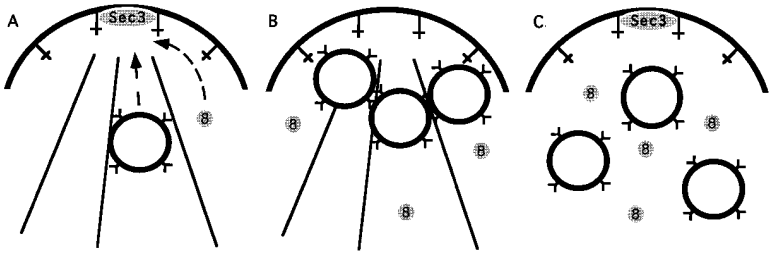

Figure 5. Model for Roles of Sec3p and Actin in Polarized Secretion

(A) In the wild-type cells, Sec3p localizes to a domain of the plasma membrane independent of the actin cytoskeleton, defining the site of exocytosis. Vesicles are transported to the correct region of the cell on actin cables. If both Sec3p and actin are functional, other factors such as Sec8p are recruited to this site, and v- and t-SNARE interaction can occur, allowing vesicle fusion.

(B) In a *sec3* mutant, vesicles are brought by actin cables to the correct region of the cell, but fusion does not occur.

(C) When actin function is lost, Sec3p is correctly localized; however, vesicles are not transported to the correct region of the cell, preventing both recruitment of Sec8p and formation of SNARE pairs. Fusion of vesicles with the plasma membrane occurs inefficiently and at inappropriate sites.

all have cell-cycle blocks at cytokinesis (Hartwell, 1971). The cells become hyperpolarized and continue to display bud tip growth, rather than switching to isotropic bud growth, and then cytokinesis (Adams and Pringle, 1984). After a 15 min shift to the restrictive temperature, Sec3-GFP was still localized to mother-bud necks (Figure 4B). The septins themselves and proteins such as Bud3p and Bud4p that bind to the septins no longer localize to the neck following a temperature shift of this length (Chant et al., 1995; Sanders and Herskowitz, 1996). Thus, Sec3-GFP is not localized to the mother-bud neck at cytokinesis through direct interaction with septins. Following a 2 hr shift to the restrictive temperature of 37°C, as expected, the Sec3-GFP was strongly concentrated at the tips of the hyperpolarized cells, demonstrating that the Sec3-GFP localization is responsive to the cell-cycle arrest imposed by the septin mutants (Figure 4B).

Sec3-GFP Localization in Cell-Cycle Mutants

The localization of both the actin and septin cytoskeletons is controlled by the polarity establishment proteins, including the rho-family GTPase Cdc42p, its exchange protein, Cdc24p, and its GTPase activating protein, Bem3p (reviewed in Drubin and Nelson, 1996; Pringle et al., 1995). Because Sec3-GFP localization was not dependent on either actin or septin function, we observed Sec3-GFP localization in *cdc24-4*, *cdc42-1*, and *bem3-1* ts mutant strains. Even after a 4 hr shift to 37°C, the Sec3-GFP remained localized as a patch on the surface of the enlarged unbudded cells (Figure 4C, Table 1). Whether this patch of Sec3-GFP is a remnant of an old cytokinesis site formed prior to the imposition of the cell-cycle block or represents the Sec3-GFP localizing to a new, but unused, bud site is not apparent.

The cyclin-dependent kinase Cdc28p regulates progression through the cell cycle through its interaction with different cyclins (reviewed in Lew et al., 1997). The localization of Sec3-GFP was examined in the ts *cdc28-1* mutant (Figure 4D). After a 15 min shift to the restrictive temperature, no apparent changes in localization were observed. By 30 min at restrictive temperature, there was an accumulation of oblong unbudded cells with a concentration of Sec3-GFP along one edge. This concentration of Sec3-GFP disappeared by 1 hr, indicating the dependence of Sec3-GFP on Cdc28p in establishing and maintaining its polarized localization.

Discussion

This study establishes Sec3p as a key component of the machinery specifying the spatial regulation of exocytosis. The localization of Sec3p changes with the cell cycle but is independent of both the cytoskeleton and continued flux through the secretory pathway. In contrast, vesicle delivery is dependent on the actin cytoskeleton. We propose that exocytosis at specific sites requires two systems working in parallel: an actin-dependent system for the transport of vesicles to the proper region of the cell, and a vesicle receiving station whose localization is actin-independent (Figure 5).

Previous work established that in addition to blocking exocytic transport (Novick et al., 1980; Finger and Novick, 1997), *sec3* mutants are defective in polarity establishment in diploids (Haarer et al., 1996; Finger and Novick, 1997). *sec3* mutants also display cytokinesis defects (Finger and Novick, 1997), partially delocalized deposition of chitin on the cell surface (Finger and Novick, 1997; Mondesert et al., 1997), and abnormalities of the actin cytoskeleton (Haarer et al., 1996; Finger and Novick, 1997). These defects could be caused by mutations in a protein necessary for establishment of sites of polarized exocytosis. The data presented here demonstrate a role for Sec3p in helping to define the site of polarized exocytosis.

First, Sec3-GFP localizes to the site of polarized exocytosis at each cell-cycle stage in haploid and diploid yeast growing vegetatively and in haploid yeast responding to mating pheromone. The changes in localization of Sec3-GFP occur synchronously with changes in the actin cytoskeleton, implying that these proteins are coordinately regulated by cell-cycle events. The localization to both ends of diploid yeast of Sec3-GFP and Sec4p implies that secretion actively occurs at both regions and could explain the more elongated lemon shape compared to haploids. The persistence of components of the secretory pathway at both proximal and distal sites also supports the hypothesis that components of the secretory pathway may be important in recognition of the distal pole in bud-site selection (Chant and Pringle, 1995).

Second, the localization of Sec3-GFP does not require any other *SEC* gene products tested, demonstrating that Sec3-GFP localization is independent of vesicle production and that the Sec3p can stably associate with the vesicle docking/fusion sites on the plasma membrane.

Furthermore, the independence of Sec3-GFP localization from the secretory pathway as a whole, including the other components of the Sec3/5/6/8/10/15/Exo70p complex (with the possible exception of Exo70p, for which no mutant is available), suggests that Sec3p may be the most membrane-proximal member of this complex and that it could be required for other components to assemble at the vesicle-docking/fusion site.

The complete independence of Sec3-GFP localization from the secretory pathway is in sharp contrast with the requirements of two other *SEC* gene products with which Sec3-GFP colocalizes. First, Sec4p, the rab family GTPase that acts in post-Golgi secretion in yeast (Salminen and Novick, 1987), has been shown by indirect immunofluorescence to require production of post-Golgi vesicles in order to localize so that any block in transport upstream of this step results in loss of the polarized distribution of Sec4p (Walch-Solimena et al., 1997). Sec4p has been shown by immuno-EM to reside on post-Golgi vesicles, explaining the loss of Sec4p staining when no vesicles are produced (Walch-Solimena et al., 1997). The polarized localization of Sec4p is dependent on one other post-Golgi *SEC* gene product, Sec2p, which is the guanine nucleotide exchange protein for Sec4p (Walch-Solimena et al., 1997). The requirements for localization of Sec8p have now been studied using indirect immunofluorescence of Sec8-3Xmyc. Surprisingly, this component of the Sec3/5/6/8/10/15/Exo70p complex is dependent on all *SEC* gene products tested for its localization, indicating that Sec8p may be recruited to vesicle fusion sites in response to vesicle docking. The requirement for all other post-Golgi *SEC* gene products may reflect the action of Sec8p at a very late stage of vesicle docking/fusion. Sec5-3XHA has recently also been localized to similar sites of polarized growth (Mondesert et al., 1997).

The role of actin in secretion has been the subject of considerable speculation and experimentation since the demonstration that ts actin mutants display loss of polarity of secretion and accumulation of post-Golgi secretory vesicles (Novick and Botstein, 1985), indicating that actin facilitates exocytosis. More recently, both Sec4p and Sec8-3Xmyc have been shown to require actin to achieve a polarized localization (Ayscough et al., 1997), and in the case of Sec4p, actin is required for maintenance of a polarized distribution (Walch-Solimena et al., 1997). We report here that Sec8-3Xmyc also requires actin for maintenance of a polarized distribution. Sec3p, in contrast, is able to maintain localization in the absence of any polarized actin and can also localize to new sites. This demonstrates that development of a polarized secretory pathway in yeast requires both actin-independent and actin-dependent steps.

Although Sec3-GFP localizes to the mother-bud neck at cytokinesis, it does not colocalize with or appear dependent on septins. Most surprisingly, Sec3-GFP appears to maintain a polarized distribution even after several hours at the restrictive temperature in the polarity establishment mutants *cdc24-4*, *cdc42-1*, and *bem3-1*, which establish the polarity of both the actin and septin cytoskeletons (Pringle et al., 1995). We have been unable to determine whether the concentration of Sec3-GFP represents remnants of old sites of polarized growth or localization to new bud sites in the absence of polarized localization of other components necessary for bud establishment. It is therefore unclear whether Sec3p is dependent on Cdc42p or is directly responding to some more upstream component of the cell-cycle machinery. The localization of Sec3-GFP is lost within 1 hour at the restrictive temperature in a *cdc28-1* mutant, suggesting that the latter may be the case.

The data presented here, in combination with previous work, suggest a model for polarized secretion whereby exocytic vesicles are transported into the bud tip in an actin-dependent manner, perhaps by transport along actin cables. Sec3p localizes to the sites of polarized exocytosis in an actin-independent manner. The coupling of actin-dependent vesicle targeting with Sec3p-dependent establishment of the fusion site allows for recruitment of other necessary factors, such as Sec8p, and allows the interaction of v- and t-SNAREs, thereby promoting membrane fusion. A homolog of the Sec3/5/6/8/10/15/Exo70p complex has been isolated from mammalian brain (Ting et al., 1995; Hsu et al., 1996), suggesting that an analogous process occurs during polarized transport in mammalian cells, in which a Sec3p homolog would play a similar role. In animal cells, a two-step process of polarized secretory vesicle transport has been described (Bi et al., 1997). The first step is kinesin-dependent transport along microtubules to the cell periphery. Yeast exocytosis would be more analogous to the second step, involving fast myosin-dependent transport near the cell membrane. A key question for future research is to understand how Sec3p, which is not predicted to be a transmembrane protein or to have any posttranslational modifications that would confer the ability to bind membranes, associates with the plasma membrane. Such studies should provide insight into the development of polarity in all eukaryotic cells.

Experimental Procedures

Yeast Strains and Growth Conditions

Standard methods were used for growth, transformation, and genetic manipulation of *S. cerevisiae* (Guthrie and Fink, 1991). Strains are listed in Table 2.

Strain Construction

Two plasmids were constructed: one, which placed the coding sequence of GFP in frame with the 3′ end of the *SEC3* coding sequence, and the other, which replaced the *SEC3* coding sequence with that of GFP. In both cases, the *SEC3* promoter and terminator were used. The coding sequence of GFP (S65G, S72A) (Cormack et al., 1996) was amplified by the polymerase chain reaction (PCR), using Vent polymerase (New England Biolabs), to introduce two *Bam*HI sites; one site was immediately 3′ to the initiating ATG, and the other was immediately 5′ to the stop codon. The amplified *GFP* sequence was digested with *Bam*HI, then subcloned into *Bam*HI-digested pUC118. The *GFP* sequence was verified by the Keck Foundation DNA Sequencing Laboratory at Yale University.

A pRS316-based *CEN URA3* plasmid (Sikorski and Hieter, 1989) containing the *SEC3* gene from the *Sca*I site upstream of the *SEC3* promoter to the *Apa*I site downstream of the terminator (3ScaI-ApaIpNB402) was mutagenized according to the Kunkel method (Kunkel et al., 1987) with the Bio-Rad Muta-gene kit to create either a single *Bgl*II site immediately 5′ to the stop codon or to create two *Bgl*II sites, one immediately 3′ to the initiating ATG and one immediately 5′ to the stop codon. When digested with *Bgl*II, the construct with one *Bgl*II site allowed insertion of the *Bam*HI-digested

Table 2. Strains Used

Strain	Genotype
NY13[a]	*MAT***a** *ura3-52*
NY279[a]	*MAT***a** *ura3-52 act1-3*
NY410[a]	*MAT***a** *ura3-52 sec8-9*
NY405[a]	*MAT***a** *ura3-52 sec4-8*
NY414[a]	*MAT***a** *ura3-52 sec13-1*
NY648[a]	*MAT***a**/*MAT*α *ura3-52/ura3-52 leu2-3, 112/leu2-3,112*
NY1356[a]	*MAT***a** *ura3-52 his7 ade2 trp1-289 lys2 cdc12-1*
NY1358[a]	*MAT***a** *ura3 his3 trp1 leu2 lys*$^{+/-}$ *CDC3::3XHA*
NY1362[a]	*MAT***a**/*MAT*α *ura3-52/ura3-52 his4-619/HIS4 sec3-2/sec3-2 GAL*$^{+}$*/gal*$^{-}$
NY1372	*MAT***a** *ura3-52 leu2-3,112::(LEU2 SEC8-3XMYC) sec3-2*
NY1373	*MAT*α *ura3-52 leu2-3,112::(LEU2 SEC8-3XMYC) sec4-8*
NY1377[a]	*MAT*α *ura3-52 leu2-3,112::(LEU2 SEC8-3XMYC)*
NY1438	*MAT***a** *ura3-52 leu2-3,112::(LEU2 SEC8-3XMYC) sec13-1*
NY1442[a]	*MAT***a** *ura3-52 leu2 his3 cdc24-4*
NY1443[a]	*MAT*α *ura3-52 leu2 trp1 cdc42-1*
NY1449[a]	*MAT*α *ura3-1 ade2-1 ade3-22 his3-11 leu2-3,112 trp1-1 can1-100 cdc28-1*
NY1583	*MAT***a** *ura3-52 leu2-3,112::(LEU2 SEC8-3XMYC)*
FY110[a]	*MAT***a** *ura3-52 sec3-4*
FY140	*MAT***a** *ura3-52 leu2-3,112 sec3::(SEC3-4XHA URA3)*

[a] Strain was transformed with pSEC3-GFP3-1.

GFP to create a fusion protein of Sec3p with GFP at its carboxyl terminus (Sec3-GFP), produced under the control of the *SEC3* promoter. This plasmid was called pSEC3-GFP3-1. The other construct, pGFP3-1, allowed the entire *SEC3* open reading frame to be replaced with that of *GFP*, enabling production of GFP under the control of the *SEC3* promoter. Growth of all strains containing either of the two plasmids was monitored at 25°C, 30°C, 34°C, and 37°C to demonstrate complementation of *sec3* temperature-sensitive mutants and to confirm that the *SEC3-GFP* had no effects on growth of other strains.

The genomic copy of *SEC3* was *GFP*-tagged as follows: the 2.1 kb *Hin*dIII-*Kpn*I fragment of pSEC3-GFP3-1 was cloned into pRS306 (Sikorski and Hieter, 1989) to create pI-SEC3-GFP3. The plasmid was linearized with *Bst*EII and transformed into yeast, where *SEC3-GFP* was able to function as the sole copy of *SEC3*. The 2.1 kb *Hin*dIII-*Kpn*I fragment of pSEC3-GFP3-1 was also used to replace the 1.4 kb *Hin*dIII-*Kpn*I fragment of 3ScaI-ApaIpNB419 (Finger and Novick, 1997) to create pHC-SEC3-GFP3-1, a yeast 2μ shuttle vector used in studies of Sec3-GFP overproduction.

Sec3p was tagged with four HA epitopes at the C-terminal region of the genomic *SEC3* gene as follows: first, the 3′ end of the *SEC3* gene, with the *Bgl*II site immediately 5′ to the stop codon, from the *Hind*III site to the *Kpn*I site in the polylinker of the plasmid was cloned into pRS306 (Sikorski and Hieter, 1989), a yeast integrating vector containing *URA3* as a selectable marker. A plasmid containing four consecutive HA epitopes (gift of Li-Lin Du) was used as the template for PCR amplification with Vent polymerase (NEB). The PCR product contained in-frame *Bam*HI sites and was subcloned into the *Bgl*II-digested *SEC3* plasmid. The resulting plasmid (pSEC3-4XHA) was linearized with *Bst*EII and transformed into yeast, where it was able to function as the sole copy of *SEC3*.

Staining of Sec3-GFP Expressing Cells with Rhodamine-Phalloidin

Cells from exponential cultures grown in dropout medium lacking uracil or in minimal medium (supplemented as necessary) were fixed and stained as described (Finger and Novick, 1997).

Fixation of Sec3-GFP Containing Cells for Temperature Shift Experiments

Cells from exponential cultures grown in dropout medium lacking uracil were fixed as follows: cells were pelleted 2 min at 4°C at 3000 rpm in a Beckman tabletop centrifuge, then fixed in phosphate-buffered saline (PBS) containing 2% glucose, 20 mM EGTA, and 3.7% formaldehyde for 1 hr at room temperature. Cells were washed twice with PBS, then permeablized with 0.1% Triton X-100 for 10 min to reduce autofluorescence. Cells were washed five times with PBS and resuspended in a small volume of PBS. For colocalization with F-actin, fixation and staining with TRITC-phalloidin (Sigma) was performed as described (Finger and Novick, 1997). For quantitation of polarized Sec3-GFP in mutants, cells were shifted to 37°C for 1 hr, fixed and permeabilized as above, mounted on slides, and Z-series collected with a Bio-Rad confocal microscope. A cell was considered to have polarized Sec3-GFP if a distinct concentration of Sec3-GFP was visible at the bud tip, mother-bud neck, or prebud site and as unpolarized if there was no detectable Sec3-GFP concentration and the Z-series had clearly sectioned through the entire cell. Cells were excluded from the calculations if no polarized concentration of Sec3-GFP was detected and the Z-series did not clearly include the entire cell or if autofluorescence prevented detection of the GFP signal. The minimum number of cells counted was 168.

For some experiments, cells shown were pelleted 1 min at 4°C, resuspended in PBS, pelleted again, then fixed 5 min in −20°C methanol, then 30 s in −20°C in acetone, and rehydrated by washing 5× with PBS. Localization of Sec3-GFP was the same in all experiments with either fixation.

Experiments where cells were α-factor arrested were performed as follows: cells from early exponential cultures in SC-ura were pelleted 2 min at 4°C at 3000 rpm in a Beckman tabletop centrifuge, washed in SC-ura (pH 4.0), and resuspended at an A_{600nm} of 0.5 in SC-ura (pH 4.0) containing 12.9 μM α-factor. Cultures were incubated at 25°C for 2–3 hr until at least 80% of cells were unbudded or shmooed, determined by classifying the morphology of 200 cells. Cells were then pelleted at 3000 rpm in a Beckman tabletop centrifuge and either fixed and stained with TRITC-phalloidin as described or washed with SC-ura, repelleted, and resuspended in SC-ura. Ts mutants were shifted to 25°C or 37°C for 30 min or 1 hr, then fixed and processed for TRITC-phalloidin staining.

Indirect Immunofluorescence of other Proteins in Sec3-GFP-Expressing Cells

Cells were pelleted 2 min at 4°C in a Beckman tabletop centrifuge, supernatants were removed, and cells were then fixed for 2 hr at room temperature in PBS containing 2% glucose, 20 mM EGTA, and 3.7% formaldehyde. Cells were washed twice with 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5), then cell walls were digested in 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5) containing 28 mM 2-mercaptoethanol and 40 μg/ml zymolyase 100T (Seikagaku) for 15–30 min at 37°C. Cells were pelleted 2 min at 4°C at 1700 rpm, then resuspended in 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5). Cell suspensions were placed on poly-L-lysine coated multiwell slides for 10 min, then unattached cells were aspirated off. Cells were permeabilized with either 0.025% SDS (for Sec4p and Cdc3p staining) for 5 min at room temperature or with −20°C methanol for

5 min, followed by −20°C acetone for 30 s (for Cdc3p and actin staining). The slides were then washed 10 times with drops of PBS containing 1% BSA (PBS-BSA) and blocked for 30 min in PBS-BSA. Slides were then incubated with primary antibody diluted in PBS-BSA (1:2000 monoclonal anti-Sec4p ammonium sulfate cut; 1:5000 monoclonal anti-HA nutridoma 12CA5 diluted 1:5000 for detection of Cdc3-3XHA, 9E10 ascites diluted 1:1000 for detection of Sec8-3Xmyc; 1:500 ICN monoclonal anti-actin MAbC4) for 1 hr at room temperature. The wells of the slides were washed with PBS-BSA 10 times and then were incubated for 1 hr at room temperature with secondary antibody diluted in PBS-BSA (1:250 Jackson Labs Texas-red conjugated goat-anti-mouse IgG). Wells were washed 10 times with PBS-BSA, and slides were mounted with Mowiol (Aldrich) and allowed to set overnight before viewing with a Bio-Rad confocal microscope.

Indirect Immunofluorescence of Sec8-3Xmyc and Sec3-4XHA

To enrich for small-budded cells where Sec8-3Xmyc was most easily detected, cells grown in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) at 25°C were synchronized by mating factor arrest as described above, except that the medium used was YPD (pH 4.0). Cells were then washed, resuspended in fresh YPD, and allowed to recover from the mating factor arrest for 45 min at 25°C. The cells were then either fixed as described above for indirect immunofluorescence, or they were shifted to 37°C for 15 min or 1 hr and then fixed. Quantitation was performed by counting photographed small-budded cells; only those cells where a bud was clearly visible were included in the calculations. At least 25 cells were counted for these experiments.

Isolation of G_0 arrested cells and treatment with LAT-A were performed as described except that since yeast were haploid, 200 μM latrunculin-A in DMSO was used (Ayscough et al., 1997). To quantitate the number of cells with polarized Sec3-4XHA, 100 cells from each 2 hr time point were counted while being viewed in the micrscope. Only those cells with a clear spot of Sec3-4XHA at one end of the cell were counted as polarized.

Indirect immunofluorescence was performed essentially as described above with the following exceptions: fixed cells were permeabilized with 0.5% SDS; primary antibody for the HA epitope of Sec3-4XHA was 12CA5 nutridoma at 1:1000 dilution in PBS-BSA; and secondary antibody was Cy3-conjugated goat anti-mouse IgG (Jackson Labs) diluted 1:250 in PBS-BSA. Cells were observed on a Zeiss Axiophot microscope as described below.

Microscopy

Epifluorescence microscopy was done with a Zeiss Axiophot microscope fitted with a 63× or a 100× immersion objective (1.3 N.A.) and standard filter cube sets. Photography was done with Kodak TMAX film (ASA 400).

Confocal microscopy was done with a Bio-Rad MRC600 confocal system attached to a Zeiss IM inverted microscope equipped with a Nikon 100X lens (1.4 N.A.). The images were collected using Kalman averaging of two scans for each time point. To minimize the drift of yeast over time, cells were usually suspended in a mixture of medium and 0.5% low-melt agarose. To quantitate the GFP signals, the instrument gain was adjusted such that yeast not expressing GFP could not be imaged, and then Z-series were collected through Sec3-GFP expressing cells at 0.2 μm intervals. To photobleach yeast, line scans were conducted through the cell body with the full laser (15 mW) power. Recovery was monitored with 10% laser scans of the field every 60 s. For quantitation of the proportion of mutants with polarized Sec3-GFP, Z-series were collected through cells with 0.5 μm steps. Images were analyzed with NIH Image or IPlabs software, and the figures were created with Adobe PageMaker. Manipulations of the images were confined to adjusting contrast and brightness and the addition of markers. Movies of the time series are available at http://info.med.yale.edu/ophtha/thom/thom.html.

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References

Adams, A.E.M., and Pringle, J.R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. J. Cell Biol. *98*, 934–945.

Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D.G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. J. Cell Biol. *137*, 399–416.

Bi, G.-Q., Morris, R.L., Liao, G., Alderton, J.M., Scholey, J.M., and Steinhardt, R.A. (1997). Kinesin- and myosin-driven steps of vesicle recruitment for Ca^{2+}-regulated exocytosis. J. Cell Biol. *138*, 999–1008.

Bowser, R., and Novick, P. (1991). Sec15 protein, an essential component of the exocytic apparatus, is associated with the plasma membrane and with a soluble 19.5*S* particle. J. Cell Biol. *112*, 1117–1131.

Bowser, R., Muller, H., Govindan, B., and Novick, P. (1992). Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that may function downstream of Sec4p to control exocytosis. J. Cell Biol. *118*, 1041–1056.

Brennwald, P., Kearns, B., Champion, K., Keränen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell *79*, 245–258.

Byers, B. (1981). Cytology of the yeast life cycle. In The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, J.N. Strathern, E.W. Jones, and J. R. Broach, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 59–96.

Chant, J., and Pringle, J.R. (1995). Patterns of bud site selection in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. *129*, 751–765.

Chant, J., Mischke, M., Mitchell, E., Herskowitz, I., and Pringle, J.R. (1995). Role of Bud3p in producing the axial budding pattern of yeast. J. Cell Biol. *129*, 767–778.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS optimized mutants of the green fluorescent protein (GFP). Gene *173*, 33–38.

Drubin, D.G., and Nelson, W.J. (1996). Origins of cell polarity. Cell *84*, 335–344.

Farkas, V., Kovarik, J., Kosinova, A., and Bauer, S. (1974). Autoradiographic study of mannan incorporation into the growing cell wall of *Saccharomyces cerevisiae*. J. Bacteriol. *117*, 265–269.

Field, C., and Schekman, R. (1980). Localized secretion of acid phosphatase reflects the pattern of cell surface growth in *Saccharomyces cerevisiae*. J. Cell Biol. *86*, 123–128.

Finger, F.P., and Novick, P. (1997). Sec3p is involved in secretion and morphogenesis in *Saccharomyces cerevisiae*. Mol. Biol. Cell *8*, 647–662.

Goodson, H.V., Anderson, B.L., Warrick, H.M., Pon, L.A., and Spudich, J.A. (1996). Synthetic lethality screen identifies a novel yeast myosin I gene (*MYO5*): myosin I proteins are required for polarization of the actin cytoskeleton. J. Cell Biol. *133*, 1277–1291.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell *53*, 753–768.

Govindan, B., and Novick, P. (1995). Development of cell polarity in budding yeast. J. Exp. Zool. *273*, 401–424.

Govindan, B., Bowser, R., and Novick, P. (1995). The role of Myo2, a yeast class V myosin, in vesicular transport. J. Cell Biol. *128*, 1055–1068.

Guo, W., Roth, D., Gatti, E., De Camilli, P., and Novick, P. (1997). Identification and characterization of homologues of the exocyst component Sec10p. FEBS Lett. *404*, 135–139.

Guthrie, C., and Fink, G.R. (1991). Guide to Yeast Genetics and Molecular Biology. Meth. Enzymol. *194*.

Haarer, B.K., Lillie, S.H., Adams, A.E., Magdolen, V., Bandlow, W., and Brown, S.S. (1990). Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. J. Cell Biol. *110*, 105–114.

Haarer, B.K., Corbett, A., Kweon, Y., Petzold, A.S., Silver, P., and Brown, S.S. (1996). *SEC3* mutations are synthetically lethal with profilin mutations and cause defects in diploid-specific bud-site selection. Genetics *144*, 495–510.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast IV: genes controlling bud emergence and cytokinesis. Exp. Cell Res. *69*, 265–276.

Hazuka, C.D., Hsu, S.C., and Scheller, R.H. (1997). Characterization of a cDNA encoding a subunit of the rat brain rsec6/8 complex. Gene *187*, 67–73.

Heim, R., Prasher, D.C., and Tsien, R.Y. (1994). Wavelength mutations and post-translational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA *91*, 12501–12504.

Hsu, S.-C., Ting, A.E., Hazuka, C.D., Davanger, S., Kenny, J.W., Kee, Y., and Scheller, R.H. (1996). The mammalian brain rsec6/8 complex. Neuron *17*, 1209–1219.

Johnson, D.I., and Pringle, J.R. (1990). Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J. Cell Biol. *111*, 143–152.

Johnston, G.C., Prendergast, J.A., and Singer, R.A. (1991). The *Saccharomyces cerevisiae* MYO2 gene encodes an essential myosin for vectorial transport of vesicles. J. Cell Biol. *113*, 539–551.

Kee, Y., Yoo, J.-S., Hazuka, C.D., Peterson, K.E., Hsu, S.-C., and Scheller, R. (1997). Subunit structure of the mammalian exocyst complex. Proc. Natl. Acad. Sci. USA *94*, 14438–14443.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Meth. Enzymol. *154*, 367–382.

Lew, D.J., Weinert, T., and Pringle, J.R. (1997). Cell cycle control in *Saccharomyces cerevisiae*. In The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Cell Cycle and Cell Biology, Volume *3*, J.R. Pringle, J. Broach, and E.W. Jones, eds. (Plainview, New York: Cold Spring Harbor Laboratory), pp. 607–695.

Li, R., Zheng, Y., and Drubin, D.G. (1995). Regulation of cortical actin assembly during polarized growth in budding yeast. J. Cell Biol. *128*, 599–615.

Longtine, M.S., DeMarini, D.J., Valencik, M.L., Al-Awar, O.S., Fares, H., De Virgilio, C., and Pringle, J.R. (1996). The septins: roles in cytokinesis and other processes. Curr. Opin. Cell Biol. *8*, 106–119.

Mondesert, G., Clarke, D.J., and Reed, S.I. (1997). Identification of genes controlling growth polarity in the budding yeast *Saccharomyces cerevisiae*: a possible role of *N*-glycosylation and involvement of the exocyst complex. Genetics *147*, 421–434.

Mulholland, J., Wesp, A., Riezman, H., and Botstein, D. (1997). Yeast actin cytoskeleton mutants accumulate a new class of Golgi-derived secretory vesicle. Mol. Biol. Cell *8*, 1481–1499.

Novick, P., and Botstein, D. (1985). Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell *40*, 405–416.

Novick, P., and Brennwald, P. (1993). Friends and family: the role of Rab GTPases in vesicular traffic. Cell *75*, 597–601.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205–215.

Pringle, J.R., Bi, E., Harkins, H.A., Zahner, J.E., Virgilio, C.D., Chant, J., Corrado, K., and Fares, H. (1995). Establishment of cell polarity in yeast. Cold Spring Harbor Sym. Quant. Biol. *60*, 729–744.

Rodriguez, J.R., and Paterson, B.M. (1990). Yeast myosin heavy chain mutant: maintenance of the cell type specific budding pattern and the normal deposition of chitin and cell wall components requires an intact myosin heavy chain gene. Cell Motil. Cytoskel. *17*, 301–308.

Salminen, A., and Novick, P.J. (1987). A *ras*-like protein is required for a post-Golgi event in yeast secretion. Cell *49*, 527–538.

Sanders, S.L., and Herskowitz, I. (1996). The Bud4 protein of yeast, required for axial budding, is localized to the mother/bud neck in a cell cycle-dependent manner. J. Cell Biol. *134*, 413–427.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for effecient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Simons, K. (1995). Biogenesis of epithelial cell surface polarity. Harvey Lect. *89*, 125–146.

TerBush, D.R., and Novick, P. (1995). Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in *Saccharomyces cerevisiae*. J. Cell Biol. *130*, 299–312.

TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. EMBO J. *15*, 6483–6494.

Ting, A.E., Hazuka, C.D., Hsu, S.-C., Kirk, M.D., Bean, A.J., and Scheller, R.H. (1995). rSec6 and rSec8, mammalian homologs of yeast proteins essential for secretion. Proc. Natl. Acad. Sci. USA *92*, 9613–9617.

Tkacz, J.S., and Lampen, J.O. (1972). Wall replication in *Saccharomyces* species: use of fluorescein-conjugated concanavalin A to reveal the site of mannan insertion. J. Gen. Microbiol. *72*, 243–247.

Tkacz, J.S., and Lampen, J.O. (1973). Surface distribution of invertase on growing *Saccharomyces* cells. J. Bacteriol. *113*, 1073–1075.

Walch-Solimena, C., Collins, R.N., and Novick, P.J. (1997). Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi secretory vesicles. J. Cell Biol. *137*, 1495–1509.

Watts, F.Z., Miller, D.M., and Orr, E. (1985). Identification of myosin heavy chain in *Saccharomyces cerevisiae*. Nature *316*, 83–85.